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# The effect of L-glutamine on the genetic transformation of embryogenic cell suspensions of gentian species (*Gentiana lutea* L., *Gentiana cruciata* L., and *Gentiana kurroo* Royle) using *Agrobacterium tumefaciens*

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**Abstract**

In this study, established embryogenic cell suspensions of three gentian species, *Gentiana cruciata* L., *Gentiana kurroo* Royle, and *Gentiana lutea* L., cultured in the presence of two media CMS1 and CMS2, were used to determine the effect of L-glutamine on the efficiency of *Agrobacterium tumefaciens*-mediated transformation. The presence of 1 $g \cdot l^{-1}$ glutamine in the co-cultivation medium and a 48-hr co-cultivation period were found to be optimal for all the cultures investigated. In order to regenerate plants in the post-transformation culture, approximately 100 mg of cell aggregates was plated as a single layer on RM1 and RM2 media. Timentin was used in post-transformation cultures for preventing bacterial contamination and enhancing cell viability. The transformants were selected in the presence of 50 $mg \cdot l^{-1}$ kanamycin. Transformation was later confirmed by histochemical analysis of the activity of reporter enzyme (β-glucuronidase) and by polymerase chain reaction for the detection of *uidA* and *nptII* genes. Five lines of embryogenic cell suspension cultures of the studied species were selected and grown in the presence of 50 $mg \cdot l^{-1}$ kanamycin. Finally, 23 embryos were regenerated, of which only 11 converted into T0 transformants of *G. cruciata*. These transformants continued to grow in the presence of kanamycin. A solid, dark blue coloration of their leaves confirmed stable integration and expression of the *uidA* gene. The molecular analysis of T0 plants revealed the absence of bacterial contamination. Thus, the short list of plant species that can be transformed by *A. tumefaciens* with the help of an embryogenic cell suspension is extended by the three species investigated in this study.

**Key words:** embryogenic cell suspensions, gentians, kanamycin resistance, L-glutamine, timentin, transformants

**Abbreviations**

| | | | |
|---|---|---|---|
| ACET | – acetosyringone | MS medium | – Murashige and Skoog medium (1962) |
| AS | – adenine sulfate | *nptII* gene | – neomycin phosphotransferase gene |
| BAP | – benzylaminopurine | NAA | – naphthaleneacetic acid |
| 2,4-D | – 2,4-dichlorophenoxy acetic acid | PCR | – polymerase chain reaction |
| DIC | – 3,6-dichloro-2-methoxybenzoic acid | PGR | – plant growth regulator |
| DMSO | – dimethyl sulfoxide | TIM | – timentin |
| $GA_3$ | – gibberellic acid | *uidA* (gus) gene | – glucuronidase gene |
| KIN | – kinetin | | |

## Introduction

The species belonging to the family Gentianaceae are economically important because of their pharmacological (production of very rich, specific secondary metabolites) and horticultural values (beauty of the flowers and variation in size and shape of the leaves) (Rybczyński et al. 2015). Hence, experimental botanists show continued interest in improving the quality of gentians using different biotechnological methods. Studies on *in vitro* morphogenesis of the species belonging to the family Gen-

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tianaceae have been undertaken for only a few taxa, with the most popular genus being *Gentiana* (Rybczyński et al., 2015). The manipulation of plant cells allows for creating efficient new combinations of different genes. At present, modification of the genome of a gentian requires modification of the methods used for the manipulation of its cells. The explant manipulation can be achieved using two approaches: callus formation and establishment of cell suspension. In gentians, two distinct types of callus proliferation are possible which yield non-embryogenic dedifferentiated (a source of secondary metabolites) and embryogenic liquid cell suspensions (a source of somatic embryos) (Chueh et al., 2001). The liquid cell suspension culture of the plant cells allows for any kind of manipulation. *Centaurium erythraea* was the first species of the family Gentianaceae to be studied for somatic embryogenesis in cell suspension maintained on MS medium in the presence of KIN, together with IAA and 2,4-D (Barešová and Kaminek, 1984). Species of the taxa *Gentiana* were selected more often than others for studies on somatic embryogenesis in cell suspension. Various combinations of PGRs, including 2,4-D + KIN or NAA + BAP + DIC + AS, were used for successful induction of embryogenic proliferation of a leaf blade tissue that originated from the plant axenic culture and various explants of a 10-day-old seedling (Ruffoni and Massabò, 1996; Mikuła et al., 2005; Fiuk and Rybczyński, 2008; Mikuła et al., 2011).

In the case of gentians, all traditional methods of plant cell transformation have been explored including particle bombardment and electroporation. However, the majority of the papers published thus far have only described the application of leaf tissues or seedling explants for transformation using bacteria *Agrobacterium rhizogenesis* and *Agrobacterium tumefaciens*. To date, a vector transformation system using *A. tumefaciens* has been developed for the following members of the family Gentianaceae: *Eustoma grandiflorum* (EHA115 pKIWI105 or pSW9), *Gentiana dahurica* (GV3130 pBI121), *Gentiana punctata* (C58C1 pArA4b), *Gentiana triflora* × *Gentiana scabra* cv. Polano-White (EHA 105 pEKB35SGtMADSbar or pSMAB-rolCGtMADS4), and *G. triflora* × *G. scabra* cv. Polano-White (pSM MARB-rolCproFT) and cv. Albireo (EHA 101 pSMABsCHS). Post-transformation cultures involving the regeneration of transformants of *G. triflora* × *G. scabra* hybrids were based on shoot regeneration of a callus resistant to various selection agents in the presence of TDZ and NAA (Hosokawa et al., 2000; Semeria et al., 1995; Wójcik and Rybczyński, 2015; Semeria et al., 1996; Mishiba et al., 2006; Mishiba et al., 2005; Nishihara et al., 2006; Nakatsuka et al., 2009; Sun and Meng, 2010).

In the majority of previously published studies on gentian transformation, fragments of plant material or even entire axenic plants were selected for experiments. In gentians, a very high production of antimicrobial metabolites is thought to occur in differentiated plant tissues characterized by a low rate of mitotic activity of cells. Some studies have reported that following a 7-day-long subculture (Mikuła et al., 2011; Fiuk and Rybczyński, 2008; Ruffoni and Massabò, 1996), embryogenic cell suspensions were characterized by a high rate of mitotic activity and a low production of secondary metabolites. These results gave us hope that this approach would be highly efficient for gentian transformation.

The embryogenic cell suspensions were utilized in vector transformation experiments in species belonging to different taxonomic clades, for example, *Carica papaya*, *Hordeum vulgare*, *Nicotiana tabacum*, *Santalum album*, and four species of *Taxus* taxa (Ketchum et al., 2007; Shekhawat et al., 2008; Kim et al., 2004; Wu et al., 1998; Carlos-Hilario and Christopher, 2015; Wilson et al., 2018). Owing to its economic value, special attention was paid to the transformation of embryogenic cell suspension of various cultivars of *Musa acuminata*, such as "Mas", "Rasthali", "Robusta" and "Dwarf Caven" (Ganapathi et al., 2001; Huang et al., 2007; Chee Wong et al., 2008; Ghosh et al., 2009; Chong-Rerez et al., 2012).

A number of attempts have been undertaken to increase the efficiency of transformation by the application of various factors (chemicals) such as amino acids. In these experiments, amino acids have played a dual role by directly improving the transformation efficiency, and as already described (Amstrong and Green, 1985; Chowdhary et al., 1993), supporting the somatic embryogenesis in cultures of various species belonging to different plant families (Shetty and McKersie, 1993; Malabadi and Van Staden, 2005; Zouine and Hadrami, 2007; Gerdakaneh et al., 2011; Pawar et al., 2015). Dan (2008) demonstrated that the use of antioxidants (glycine, betaine, glutathione, and polyvinylpyrrolidone) accelerated transformation. Much effort was also devoted to improving the transformation efficiency of *Citrus aurantifolia* (Dan et al., 2008) and other crops (Dong and McHughen, 1993).

There are numerous plant species in which the involvement of amino acids in induction and establishment of various types of cultured calli tissues and cell suspensions derived from them has been described. In these cases, organic nitrogen was used to support the growth of the tissues cultured in artificial (*in vitro*) conditions and to control the morphogenic processes. Amino acids such as L-alanine, L-proline, L-glutamine, L-asparagine, and L-tryptophan were used individually (Haroun et al. 2010; Gerdakaneh et al. 20110) or in combinations (Garin et al., 2000; Malabadi and van Staden, 2005). Additionally, some of the amino acids including L-glutamine have been used for improving the efficiency of *Agrobacterium* transformation.

In the case of leaf blade explants of *Gentiana cruciata*, L-glutamine was used as an essential factor for obtaining callus tissue and ensuring stable transformation. The highest number of T0 plants was regenerated in the presence of 1 $g \cdot l^{-1}$ L-glutamine (Wójcik and Rybczyński, 2017). Based on our experience with vector (Wójcik and Rybczyński, 2017) and nonvector (Wójcik and Rybczyński, 2015) transformations of gentians, complexes of (mitotically active) embryogenic cells were selected for the experiments conducted in the present study to test the effect of L-glutamine on transformation efficiency and maintenance of embryogenic potential. Thus, the aim of this study was to investigate the effects of L-glutamine on the efficiency of *Agrobacterium*-mediated transformation of the established cell suspensions of three gentian species, namely *G. cruciata*, *Gentiana kurroo*, and *Gentiana lutea*. *A. tumefaciens* strain C58C1 carrying *nptII* and *uidA* genes was used as a vector. The transformation system used in this study was based on the liquid co-culture of plant cell aggregates with bacterial cells in the presence of L-glutamine. Additional effort was made to remove bacterial cells from the suspension during all steps of the post-transformation procedure and from regeneration cultures in order to obtain a bacteria-free culture. The cultures resulted in the formation of somatic embryos and regeneration of T0 transformants. Unfortunately, we only present the description of T0 here since the environmental protection law currently binding in Poland forbids carrying out experiments on genetically modified organisms outside the laboratory. Being perennials, gentians need to be grown in field for a few years in order for them to attain sufficient development to produce next sexual generation, which is required for understanding the entire transgenesis process.

## Materials and methods

### *Cell suspension culture*

Three well-established cell suspensions of *G. cruciata* L. (cotyledon origin), *G. kurroo* Royle (cotyledon derived), and *G. lutea* L. (seedling root origin) maintained in 200 ml conical flasks were used for the experiments. Cultures were maintained in liquid MS media supplemented with (1) 0.5 $mg \cdot l^{-1}$ 2,4-D + 1 $mg \cdot l^{-1}$ KIN (CSM1) or (2) 2 $mg \cdot l^{-1}$ BAP + 1 $mg \cdot l^{-1}$ DIC + 0.1 $mg \cdot l^{-1}$ NAA + 80 $mg \cdot l^{-1}$ AS (CSM2) (Table 1). Both the media were supplemented with 30 $g \cdot l^{-1}$ sucrose, and the pH was adjusted to 5.6 before autoclaving. The cultures were placed on a rotary shaker at 120 rpm and kept in a growth chamber at 22 ±1°C under illumination of 3.5 $\mu Em^{-2}s^{-1}$. Subculturing was done weekly using cells in exponential growth. The growth of cell suspension culture was documented by photographs taken using Olympus microscopes and cameras.

### *Agrobacterium tumefaciens culture*

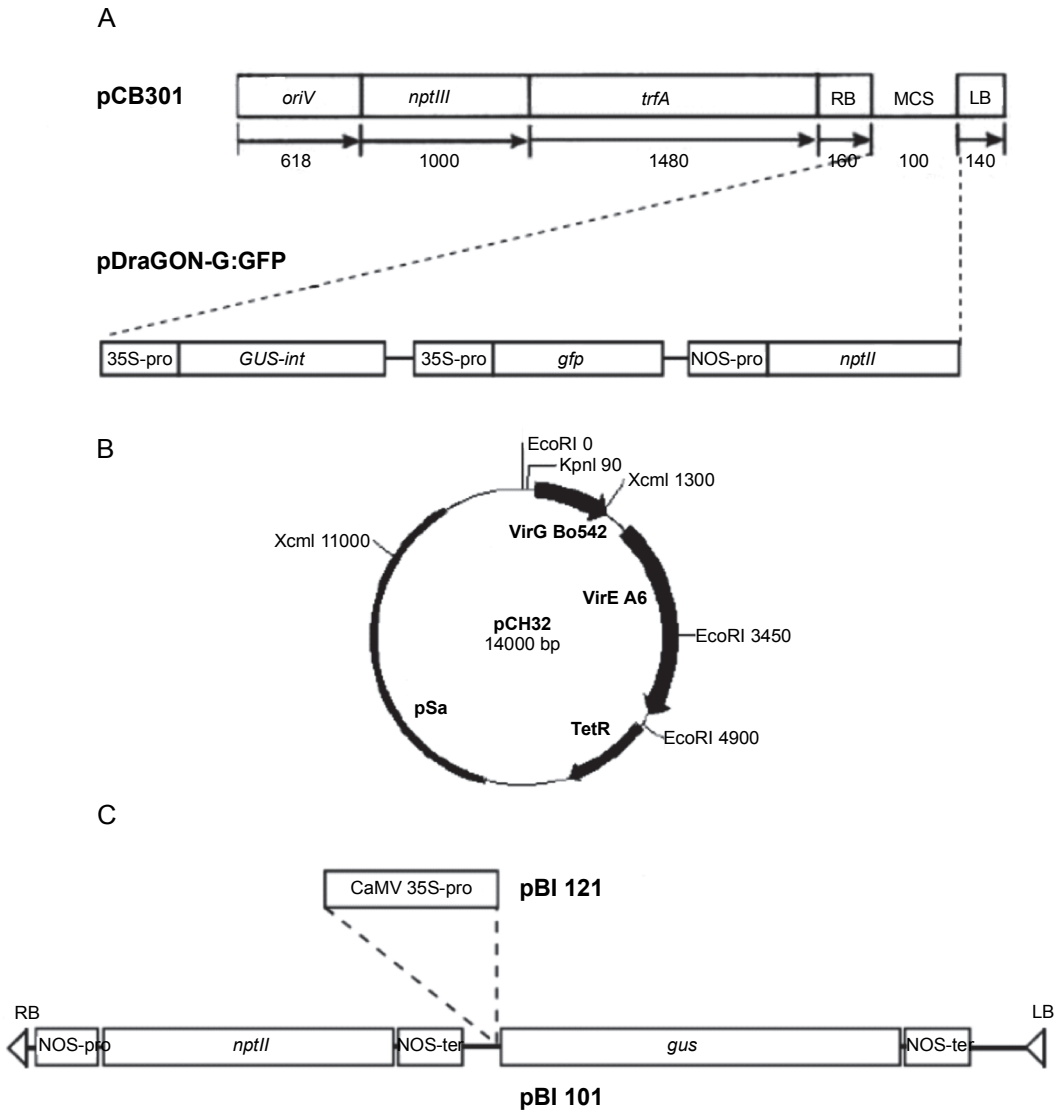
*Agrobacterium tumefaciens* cells were maintained in a YEB agar medium (5 $g \cdot l^{-1}$ peptone, 5 $g \cdot l^{-1}$ yeast extract, 1 $g \cdot l^{-1}$ beef extract, 0.49 $g \cdot l^{-1}$ $MgSO_4$ with or without 15 $g \cdot l^{-1}$ agar, pH = 7.2). Two-day-old subcultures of individual colonies of bacteria were resuspended in small volumes of YEB medium and were subsequently transferred to a fresh liquid YEB medium for multiplication. These cultures were maintained for 24 hrs in the dark at 28°C on a rotary shaker at 180 rpm. Just prior to the experiment, the bacteria were collected and washed three times using sterile 10 mM $MgSO_4$ by centrifugation. Finally, they were transferred to the inoculation medium (IM3) (Table 2) with OD = 0.8 at $\lambda$ = 600 nm.

### *Kanamycin (selection agent) concentration testing for cell aggregate elimination*

Different concentrations of kanamycin (0, 50, 100, 150, 200, and 250 $mg \cdot l^{-1}$) were tested for evaluating the survival and selection of 1 g of *G. cruciata* and *G. kurroo* cell suspension cultured in 30 ml of liquid CSM1 and CSM2 media. The experiment comprised five replicates. Four-week-old cultures were used. Visual changes and total fresh and dry weights of samples were examined for evaluating the response of cultured aggregates.

**Table 1.** Media used for cell suspension culture

| Medium | Content | Application |
|---|---|---|
| **CSM1** | MS + 0.5 mg·l$^{-1}$ 2,4-D + 1.0 mg·l$^{-1}$ KIN + 30 g·l$^{-1}$ sucrose | Media used for cell suspension culture |
| **CSM2** | MS + 2.0 mg·l$^{-1}$ BAP + 1.0 mg·l$^{-1}$ DIC + 0.1 mg·l$^{-1}$ NAA + 80 mg·l$^{-1}$ SA + 30 g·l$^{-1}$ sucrose | |




**Schema 1**. Map of the plasmids used for an efficient transformation of gentian cell suspensions. A) pCB301 (Xiang et al., 1999) and vector pDraGON-G:GFP; B) helper plasmid pCH (Hamilton, 1997) (www.biotech.cornell.edu); C) fragment of T-DNA plasmid pBI 121 (Jefferson et al., 1987) (RB/LB – right/left border; MCS – polylinker (multiple cloning site); gus – β-glucuronidase-coding gene; *nptII* and *nptIII* – neomycin phosphotransferase-coding genes (kanamycin resistance); *gfp* – green fluorescence protein-coding gene; 35S-pro (CaMV 35S pro) – Cauliflower Mosaic Virus promoter; NOS-pro/NOS-ter – promoter/terminator of nopalin synthase gene; oriV and trfA – part of ori replication of RK2 plasmid; pSa – ori replication of PSa plasmid; TetR – resistance for tetracycline; *virV* and *virG* genes – *Agrobacterium* genes responsible for vir activation and T-DNA transfer

**Table 2.** Media used for transformation of embryogenic cell suspension with *Agrobacterium tumefaciens*

| Medium | Content | Application |
|---|---|---|
| IM3 | MS + PGR used for particular cell suspension + ACET + 0; 0.25; 0.5; 1.0; 2.0 $g \cdot l^{-1}$ GLN | Media used for cell suspension incubation with *Agrobacterium* C58C1 |
| AEM1 | MS + PGR used for particular cell suspension + 30 $g \cdot l^{-1}$ sucrose + TIM | Medium for *Agrobacterium* elimination from transgenic cell suspension |
| SM1 | MS + PGR used for particular cell suspension + 30 $g \cdot l^{-1}$ sucrose + TIM + KAN | Medium for transgenic cell suspension aggregate selection |
| RM1 | MS + 1.0 $mg \cdot l^{-1}$ KIN + 0.5 $mg \cdot l^{-1}$ $GA_3$ + 80 $mg \cdot l^{-1}$ AS + 30 $g \cdot l^{-1}$ sucrose + KAN + agar | Medium for genetically modified regenerants regeneration from transformed cell suspension |
| RM2 | MS + 30 $g \cdot l^{-1}$ sucrose + KAN + agar | |
| TPM | MS + 30 $g \cdot l^{-1}$ sucrose + KAN + agar | Medium for tranformants *in vitro* culture |

For a better analysis of tissue response to kanamycin, additional experiments on agar medium were carried out only for the cell suspension of *G. cruciata* using RM1 medium (Table 2). Cell aggregates (100 mg) were plated onto medium (six replicates). Subculturing was performed fortnightly. After 8 weeks of culturing, the effect of the antibiotic on the growth of cell aggregates was evaluated by measuring the number of somatic embryos regenerated, together with the fresh weight of tissue. For subsequent subcultures on liquid and agar medium, only 50 $mg \cdot l^{-1}$ kanamycin was used for transgenesis selection.

***Effect of L-glutamine on transformation efficiency***

In order to ascertain the effect of L-glutamine on transformation of cell aggregates, filter-sterilized L-glutamine at concentrations ranging from 0 to 2 $g \cdot l^{-1}$ was used. The time of treatment of cell suspension was correlated with the time of co-culture of the plant material with bacteria for 24 and 48 hrs.

***Transformation of embryogenic cell suspension aggregates***

One gram of cell aggregates collected from the suspension on sterile nylon sieves at the beginning of the logarithmic phase of the growth curve was transferred to a conical flask (100 ml) and resuspended in 30 ml of bacterial inoculum. Co-cultures were incubated for 24 and 48 hrs. Later, cultures were washed with sucrose-free media: CSM1 or CSM2 (Table 1). Aggregates were subsequently cultured with AEM1 medium (along with TIM) for 1 week in order to eliminate the bacteria. Finally, the cultures were transferred to SM1 liquid medium for kanamycin selection. After 2 weeks of culturing, the transformation efficiency was assessed by analyzing the expression of β-glucuronidase reporter gene. Transformation efficiency was expressed in terms of number of dark and light blue-stained cell aggregates visible under a light microscope in a sample of 50 aggregates. For the next 3 months, cell suspensions were cultured under selection using 50 $mg \cdot l^{-1}$ kanamycin. Following this period (i.e. 16 weeks after the transformation experiment), the cultures were evaluated for the second time using the GUS histochemical assay and subjected to a molecular analysis.

***Post-transformation cell suspension cultures and plant regeneration (transformants T0)***

In order to aid plant regeneration in the post-transformation culture, following 4 weeks of selection, approximately 100 mg of cell aggregates was plated as a single layer on RM1 and RM2 (Table 2) agar-solidified media. Subcultures were carried out at fortnightly until somatic embryos became apparent. The conversion of somatic embryos into plantlets (germlings) was carried out on TPM medium containing kanamycin. On this medium, regenerated plantlets developed root systems and produced numerous leaves which served as material for further histochemical analyses of GUS and for PCR for confirming transformation.

***Assay of GUS activity***

The expression of GUS in cells, calluses, and leaf fragments of transformants was assayed using 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc) as substrate. Tissues were incubated in a reaction mixture comprising 1 mM of substrate in DMSO, 20 mM phosphate buffer

(pH = 7.0), 0.25 mM Triton X-100, and 3.12 mM methanol. The plant material was immersed in reaction mixture and kept at 37°C for 24 hrs in the dark. Following this, the plant material was treated with 70% ethanol three times to extract chlorophyll from tissues, so that the blue (dichloro-dibromoindigo) coloration due to GUS reaction could be seen. Finally, the tissues were fixed with Carnoy's fixative (acetic acid:ethanol in the ratio of 1:3). The expression of GUS in cells was investigated twice: at the second and the 12$^{th}$ week post-transformation. For the second investigation, only 50 aggregates of each cell suspension, which survived kanamycin selection, were used (Table 3).

### *PCR analysis of transgenesis*

The transgenic events were confirmed by carrying out PCR of both *nptII* and *uidA* genes. The following forward and reverse primers were used for *nptII* gene: 5′–GAGGCTATTCGGCTATGACTG–3′ and 5′–ATCGG GAGCGGCGATACCGTA–3′, respectively; these primers amplified a fragment of 700 bp (Wójcik and Rybczyński, 2017). The thermal profile adopted for PCR was as follows: pre-denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and elongation at 72°C for 60 sec; this was followed by a final extension at 72°C for 5 min and a pause at 4°C. For *uidA* gene, the following forward and reverse primers were used: 5′–TTATCTCTATGAA CTGTGCGTCA–3′ and 5′–TTGGACATACCATCCGTAA TAA–3′, respectively; these primers amplified a fragment of 679 bp. The primers were designed using the Primer3 program on the basis of sequences of *uidA* gene found in the NCBI database. The following thermal profile was adopted for PCR: pre-denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 51°C for 1 min, and elongation at 72°C for 2 min; this was followed by a final extension at 72°C for 10 min and a pause at 4°C.

### *Statistical analysis*

Statistical analysis was performed using Statgraphics Plus4.1 program.

## Results

### *Evaluation of the effect of kanamycin concentration on cell suspension growth*

The effect of kanamycin on the growth of cell suspension was studied at concentrations ranging between 0 and 250 mg·l$^{-1}$. The toxic effect of kanamycin on the growth and development of cells was evaluated in terms of both fresh and dry mass of culture. The lowest concentration of kanamycin (50.0 mg·l$^{-1}$) appeared to be the most critical one because it reduced the biomass by fivefold in all the investigated cell suspensions. In contrast, higher concentrations (100.0–250.0 mg·l$^{-1}$) caused quicker degradation of suspensions and appearance of necrotic spots in aggregates grown on the agar medium. Figure 1 shows the response of cell suspension of *G. cruciata* as an example of the toxic effect of kanamycin on the growth of gentian cells. The media supplemented with various PGRs did not show any increase in the survival rate even when kanamycin was present at 50.0 mg·l$^{-1}$.

### *Transient expression of gus gene in cell aggregates*

The histochemical analysis of the expression of the reporter *gus* gene in cell aggregates was used as the main tool for evaluating the transient expression in cell suspensions. One criterion that was considered for this analysis was dark *vs.* pale blue staining of cells. Following the incubation of the cell suspension with *Agrobacterium* and TIM, transformation was evaluated in the presence of the selection agent kanamycin (50 mg·l$^{-1}$) (Table 3).

### *Transformation of G. lutea cell suspension*

In the case of cell suspension of *G. lutea* (Fig. 2), the presence of L-glutamine in the co-culture medium was found to have the greatest effect on the transformation efficiency. The highest transformation frequency of 25% occurred at the concentration of 1 g·l$^{-1}$ of L-glutamine. Under this condition, a strong staining of cell aggregates due to *gus* gene expression was observed in 17% of the total scored aggregates. At lower (0.5 g·l$^{-1}$) and higher (2 g·l$^{-1}$) concentrations of glutamine, the transient expression was 20% and 21%, respectively, which was not significantly different (Fig. 3A). The strongest *gus* gene expression was observed when the medium was supplemented with 2 g·l$^{-1}$ L-glutamine; however, the aggregates did not survive the selection pressure at 50 mg·l$^{-1}$ kanamycin. Only aggregates that transformed in the presence of 1 g·l$^{-1}$ L-glutamine survived kanamycin selection and continued to show meristematic activity which led to the growth of transformed cells during the next 3 months (Table 4). Finally, only one cell line was developed.

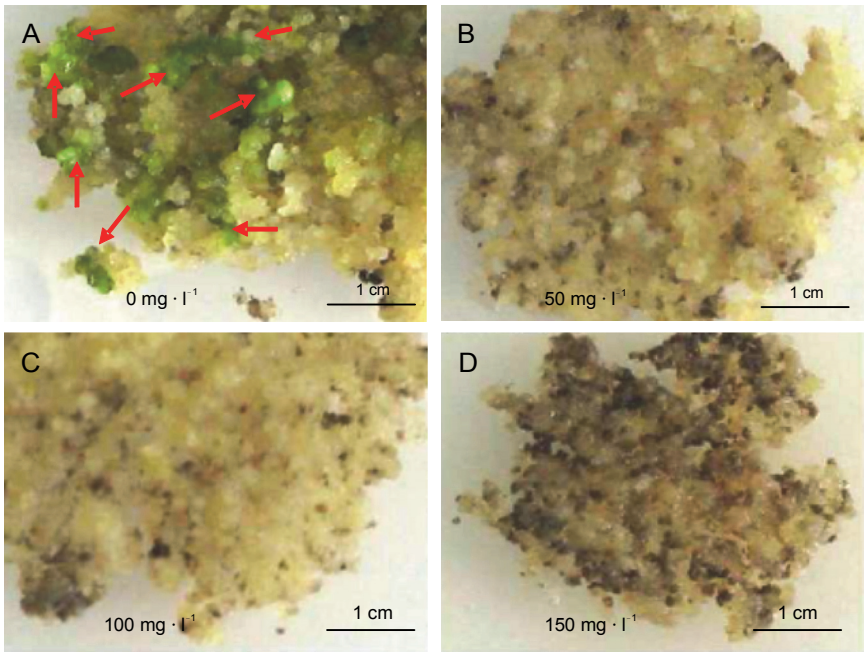



**Fig. 1.** The necrotic response of *Gentiana cruciata* cell suspension in the presence of increasing concentration of kanamycin; brown spots represent the necrotic cell aggregates on SM1 agar medium; red spots present somatic embryo formation

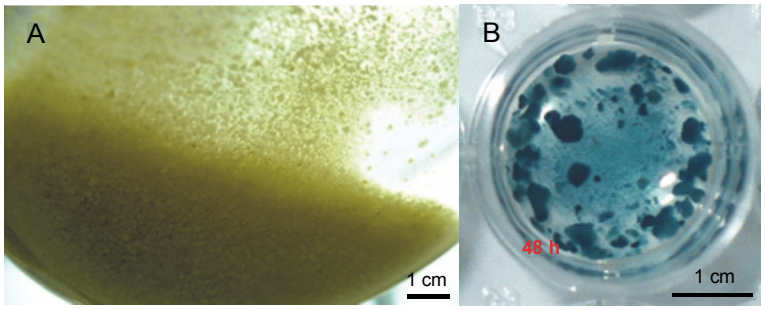



**Fig. 2.** Embryogenic cell suspension of *Gentiana lutea*. A) established 6-month-old embryogenic cell suspension in CSM1 liquid medium in conical flask; B) β-glucuronidase expression in modified line of cell aggregates observed after 3 months of culture; *Agrobacterium* transformation was done during 48 hrs in the presence of 1.0 g · l$^{-1}$ L-glutamine

***Transformation of G. kurroo cell suspension***

Cell suspensions of *G. kurroo* were found to be more sensitive to *Agrobacterium* transformation and to incubation in the absence of any transformation agent, as only 8% of cell aggregates showed a positive GUS expression. Supplementing the co-cultivation medium with 0.25 g · l$^{-1}$ L-glutamine increased the number of blue-stained aggregates, but the increase was insignificant. The most effective concentration of L-glutamine appeared to be 2 g · l$^{-1}$, at which 37% of aggregates (Fig. 3 and Fig. 3B) were successfully stained, although the differences in the staining observed between 2 and 1 g · l$^{-1}$ L-glutamine were statistically insignificant. Compared to *G. lutea*, the percentage of strongly stained aggregates was higher at both concentrations. As described for the cell suspension of *G. lutea*, only the cell line established in the presence of 1 g · l$^{-1}$ L-glutamine was able to survive the selection pressure at 50 mg · l$^{-1}$ kanamycin and develop a transgenic cell line (Table 4). Finally, only one cell line was established.

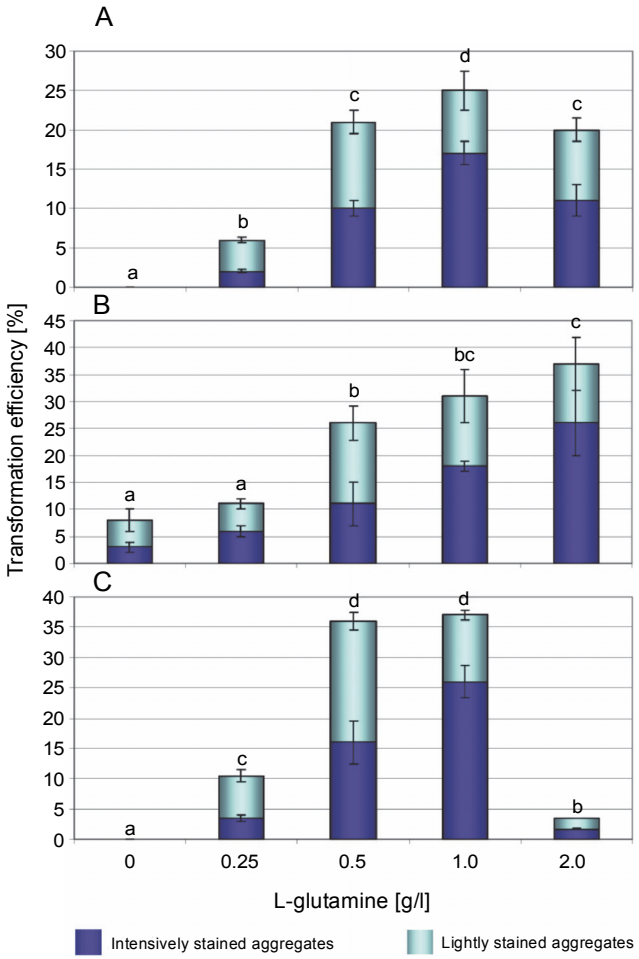



**Fig. 3.** The effect of L-glutamine on the efficiency of aggregate transformation analyzed after 14 days of culturing following transformation (the same letters are not significantly different at $P > 0.05$). A) transformation efficiency of *Gentiana lutea*; B) transformation efficiency of *Gentiana kurroo*; C) transformation efficiency of *Gentiana cruciata*

### *Transformation of G. cruciata cell suspension*

Similar to the other two cell suspensions, the amino acid L-glutamine was found to improve the transformation efficiency of cell suspension of *G. cruciata*. In the presence of L-glutamine at the four concentrations studied, the reporter gene expression was evaluated at both 24 and 48 hrs of co-culture. No transient expression of GUS was found in the control culture. The highest frequency of transformation (37%) was observed in the presence of 1 $g \cdot l^{-1}$ L-glutamine compared with 0.5 $mg \cdot l^{-1}$; however, the difference between the frequencies was insignificant. In the presence of 0.25 $g \cdot l^{-1}$ L-glutamine, only 10.5% of aggregates expressed transient blue staining, of which only 3% showed strong expression. The lowest level of transient expression (3.5%) was observed at the concentration of 2 $g \cdot l^{-1}$ of L-glutamine following 48 hrs of co-cultivation. This long duration of co-culture changed the response of cell aggregates, including the bursting of external cells and the browning of aggregates, leading to the death of the culture. The transient expression remained unaffected by various combinations of PGRs that were used to supplement the basal medium (CSM1 and CSM2) (Table 2, Fig. 3C and Fig. 5).

The size of the cell aggregates was not found to have any effect on β-glucuronidase activity. The reduction of period of exposure to L-glutamine to 24 hrs reduced the transformation efficiency from 37 to 27% at the concentration of 1 $g \cdot l^{-1}$. A significant reduction in the number of blue-stained aggregates (from 36 to 16%) was observed in the medium containing 0.5 $g \cdot l^{-1}$ L-glutamine. For both the periods of co-culture (24 and 48 hrs), a higher percentage of strong blue GUS staining was found in the presence of 1 $g \cdot l^{-1}$ L-glutamine (Fig. 3B). Shortening the incubation period improved the survival of cell aggregates. Genetically modified cell lines of *G. cruciata* showed a stable β-glucuronidase activity and were developed from cells incubated with 1 $g \cdot l^{-1}$ L-glutamine for 24 and 48 hrs or 0.5 $g \cdot l^{-1}$ L-glutamine for 24 hrs. Finally, three cell lines were established (Table 3).

### *Plant regeneration from transformed cell lines*

Many cell aggregates were found to be stained blue (evidence of transient expression), but very few produced transgenic lines. In the case of both *G. kurroo* and *G. lutea*, only one transgenic line was established. In the presence of 1 $mg \cdot l^{-1}$ KIN, 0.5 $mg \cdot l^{-1}$ $GA_3$, and 80 $mg \cdot l^{-1}$ AS + 50 $mg \cdot l^{-1}$ kanamycin (RM4 medium), the transgenic lines of *G. lutea* and *G. kurroo* lost their morphogenic potential. In the case of *G. cruciata*, three transgenic lines were established, of which only one had morphogenic potential, which was developed by incubating for 48 hrs in the presence of bacteria and 1 $mg \cdot l^{-1}$ L-glutamine. While evaluating the morphogenic potential, it was observed that 100 $mg \cdot l^{-1}$ of transgenic tissue produced 4.6 ± 1.4 somatic embryos, which showed 14-fold lower efficiency than the control culture (64.0 ± 3.2). Finally, 23 perfectly formed somatic embryos were collected from the transgenic culture. Eleven germlings or transformants were obtained on a hormone-free RM2 medium supplemented with 50 $mg \cdot l^{-1}$ kanamycin. All transformants showed *gus* expression (Fig. 5, Table 4).

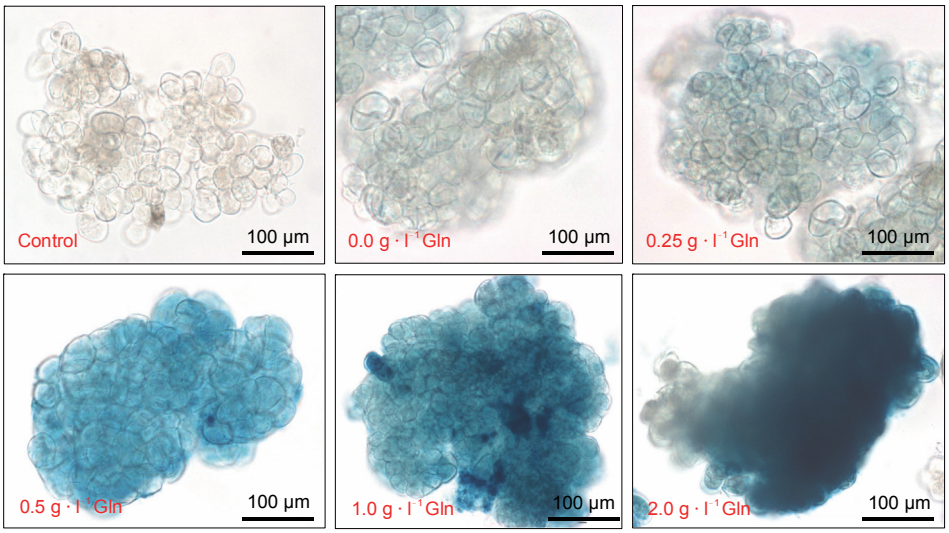



**Fig. 4.** Histochemical reaction of GUS observed after 14 days following transformation of *Gentiana kurroo* aggregates with *Agrobacterium tumefaciens* strain C58C1 after 48-hr incubation in the presence of L-glutamine at various concentrations (RM1 medium)

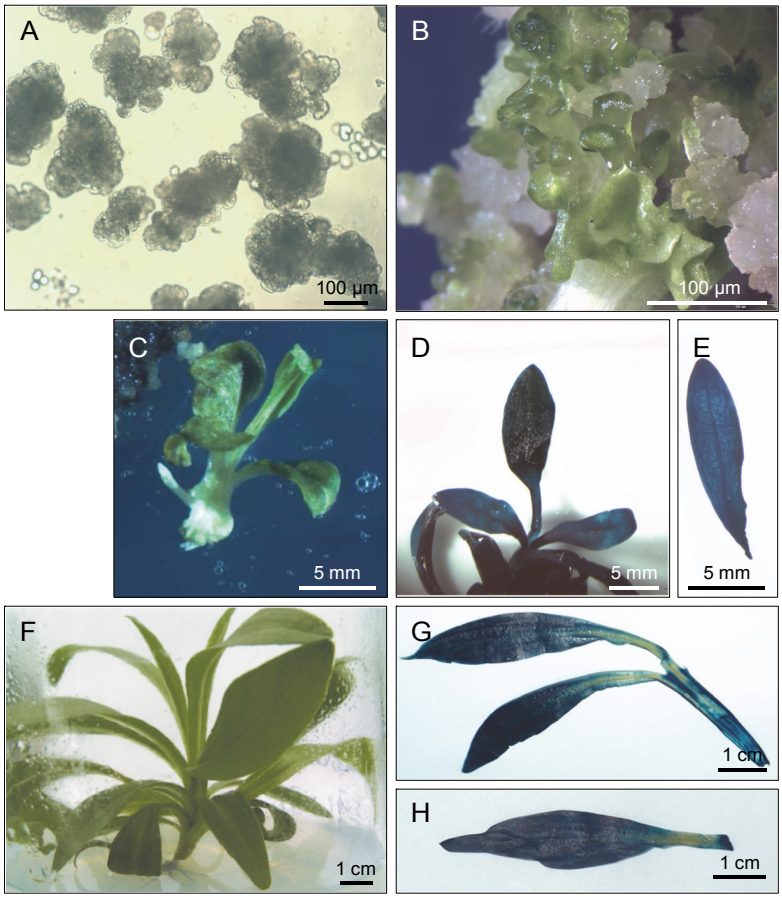



**Fig. 5.** Stages from transformed embryogenic cell suspension to T0 transformants showing β-glucuronidase expression (*Gentiana cruciata*). A) transgenic cell suspension obtained following 48-h incubation with *Agrobacterium tumefaciens* carrying C58C1 plasmid in the medium supplemented with 1.0 g · $l^{-1}$ glutamine; B) somatic embryo regeneration from transformed cell aggregates (RM1 medium); C) transformant T0 obtained from somatic embryo derived from transformed cell aggregates (RM2 medium); D) regenerated plantlet obtained in the presence of kanamycin following reaction of β-glucuronidase; E) expression of β-glucuronidase in the leaf of one of the transformantsT0; F) four-month-old transformant T0 cultured in the presence of kanamycin (RM2 medium); G) and H) intensive blue coloration indicating GUS activity of the lowest leaf and upper leaf whorls of other T0 plants

**Table 3.** GUS activity in genetically modified plant material derived from transformation experiments with *Agrobacterium tumefaciens* C58C1

| Species | Type of material | Number of cell aggregates express *gus* gene / total number | Time after transformation (age of tissue or plants) |
|---|---|---|---|
| *G. cruciata* | embryogenic cell aggregates | 3705 / 22050 | 2 weeks |
| | | 50 / 50 | 12 weeks |
| | regenerated plants | 11 / 11 | 20 weeks (12 weeks) |
| *G. kurroo* | embryogenic cell aggregates | 508 / 2250 | 2 weeks |
| | | 50 / 50 | 12 weeks |
| *G. lutea* | cell aggregates | 324 / 2250 | 2 weeks |
| | | 50 / 50 | 12 weeks |

**Table 4.** PCR analysis of genetically modified cell suspensions and derived from them plants resistant to kanamycin

| Species | Type of samples | Presence of studied genes | | | Total number of sample studied with PCR |
|---|---|---|---|---|---|
| | | *nptII* | *uid A* | *vir G* | |
| *G. cruciata* | embryogenic cell aggregates | 3 cell lines | | lack | 3 cell lines |
| | regeneration plants | 11 plantlets | | | 11 plants |
| *G. kurroo* | embryogenic cell aggregates | 1 cell line | | | 1 cell line |
| *G. lutea* | cell aggregates | 1 cell line | | | 1 cell line |

***DNA analysis of kanamycin-resistant cell suspensions and T0 plants***

The five transformed cell suspension lines which originated from the three plant species studied expressed both the *nptII* and *uidA* genes. The PCR analysis also showed the elimination of *Agrobacterium* contamination and confirmed the presence of *nptII* and *uidA* genes in all the 11 regenerated transformants. The DNA of these plants did not have the *virG* gene, and therefore, transformants were confirmed to be free of *A. tumefaciens* contamination (Fig. 6).

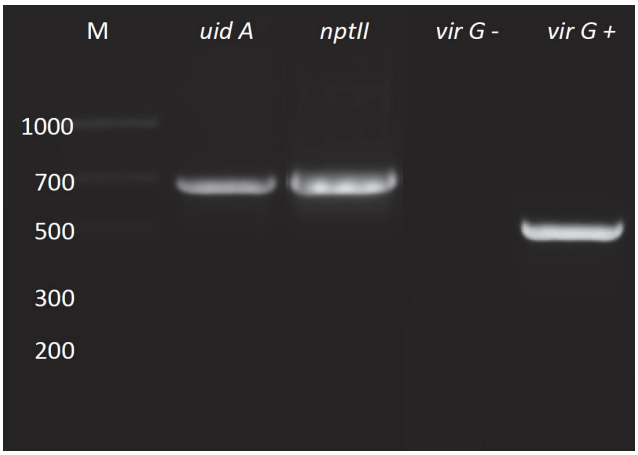



**Fig. 6.** PCR analysis of transgenic gentians which did not express *virG* gene: *virG*– – *Agrobacterium tumefaciens* contamination; *virG*+ – positive control (*A. tumefaciens* DNA)

## Discussion

The establishment and maintenance of embryogenic cell suspensions of various species of the genus *Gentiana* has been our focus for many years, as this type of culture provides an excellent material for cryopreservation of cell suspensions (Mikuła et al., 2011), protoplast culture (Fiuk and Rybczyński, 2007; Tomiczak et al., 2015), somatic hybridization (Tomiczak et al., 2015; Tomiczak et al., 2017), protoplast electroporation (Wójcik and Rybczyński, 2015), and plant gene manipulation including vector transformation. Gentians represent one of the richest sources of secondary metabolites in the plant world, and their products possess a strong and broad-spectrum antibacterial activity (Hosokawa et al., 2000). Due to this reason, *Agrobacterium* transformation of gentians appears promising for the maintenance of embryogenic potential. The present paper supplements our knowledge of the morphogenic potential of

gentian cells in stress conditions in the presence of the *Agrobacterium* co-culture. In this paper, we describe for the first time the *Agrobacterium*-mediated transformation of embryogenic cell suspensions derived from selected members of the family Gentianaceae. Until now, only one paper on transformation of proliferating cell suspension culture of gentians has been published (Hosokawa et al., 2000). In this study, the authors transformed a leaf explant-derived cell suspension of *G. triflora* × *G. scabra* using a biolistic method. The transformation parameters were examined by monitoring the transient expression of β-glucuronidase gene driven by CaMV 35S promoter. Following a two-step hygromycin (30 $mg \cdot l^{-1}$) selection in liquid and agar-solidified media, putative transformed calli were obtained and only two transgenic gentian plants were regenerated from two selected callus lines. PCR analysis and Southern blot revealed stable integration of the reporter gene (Hosokawa et al., 2000).

In this paper, we present the transformation of three suspensions characterized by morpho- and embryogenic potential using *A. tumefaciens* C58C1 strain carrying a pDraGON-G:GFP vector together with a helper plasmid pCH32 in the presence of the amino acid L-glutamine. Glutamine is one of the primary amino acids formed by nitrogen assimilation and often the most abundant amino acid found in plants (Young et al., 2010). L-glutamine works in two ways: firstly by increasing the efficiency of transformation and protecting *Agrobacterium* cells against the negative effect of plant-derived polyphenols and secondly by protecting the ability of bacteria to transfer fragments of T-DNA (Sandal et al., 2007). Amino acid inactivates the quinones, or the oxidized products of polyphenols, through replacement of its nitrogen atom by one of the oxygen atoms of quinones, thereby preventing their harmful effects (Kumar et al., 2013).

In our experiments, L-glutamine showed statistically significant effects on the transient expression of the studied cell suspensions. It was found to be an indispensable factor for the stable expression of transgenes in gentian tissues and plants. The concentrations of 0.5 and 1 $g \cdot l^{-1}$ of L-glutamine significantly influenced the transformation of all the three investigated cell suspensions. Thus, our results confirmed the findings of other studies which showed the importance of application of L-glutamine for achieving successful transformation of *Camellia sinensis* (tea), a plant that copiously produces secondary metabolites and polyphenols (Sandal et al., 2007; Mondal et al., 2001). In the case of *G. lutea* and *G. cruciata*, 0.5 and 1 $g \cdot l^{-1}$ L-glutamine seemed effective for improving transformation efficiency (Sandal et al. 2007; Mondal et al. 2001). In the case of *G. kurroo*, the transformation efficiency increased with the increase in the concentration of L-glutamine. At the concentration of 2 $mg \cdot l^{-1}$ of L-glutamine, a difference in the response of the suspensions was observed which was evident from their morphogenic potential, with *G. kurroo* showing a higher morphogenic potential.

Conversely, a transformation study of barley microspores showed that glutamine did not enhance the transformation efficiency of *A. tumefaciens*, and surprisingly, it was not required during the 2 days of co-culture during which the androgenetic development of microspores took place (Kumlehn et al., 2006). The application of other factors known to be increasing transformation efficiency, such as ascorbic acid, glutathione, and tocopherol, was totally unsuccessful in our study (data not shown). In addition, we did not examine the function of ACET (20 $mg \cdot l^{-1}$) as an obligatory factor for *Agrobacterium* transformation in the present study (Palmer and Shaw, 1992).

For all the three cell suspensions studied, 48 hrs of incubation with *A. tumefaciens* C58C1 appeared to be optimal for obtaining the greatest number of transformed cell aggregates when TIM was added to the culture. TIM is a bacteriocidal agent and prevents *Agrobacterium* from overgrowing on cells. In the case of *G. cruciata*, a cell suspension-derived transgenic line was regenerated following only 24 hrs of incubation. However, statistical analysis of the primary results indicated that 48-hr incubation of cell suspension was preferable. In contrast to other transformation systems, which are based on tissue wounding and subsequent production of phenolic compounds and sugars (Cangelosi et al., 1990), cell suspensions, without being wounded, required a longer incubation period and a longer duration of TIM treatment for the elimination of bacterial contamination. Likewise, cell suspensions of *Arabidopsis thaliana* were also co-cultured (shaken) with bacteria for 48 hrs (Gallego et al., 1999), but *Nicotiana tabacum* required as much as 72 hrs of incubation (Rempel and Nelson, 1995). The conditions necessary for *Agrobacterium* transformation of embryogenic cell suspension cultures appeared to be improved by the absence of phenols and

sugars (natural attractants of bacteria) derived from the transformed plant cells from the cultures, while ACET was the only extraneous factor that induced bacterial virulent genes thereby improving transformation (Palmer et al., 1992; Cangelosi et al., 1990). In this respect, the ultrastructure of embryogenic cell suspension aggregates revealed that the cell wall of external cells of an aggregate was several times thicker than the one formed between daughter cells inside the aggregate (Mikuła et al., 2005). Our results showed that L-glutamine plays an important role like other amino acids (e.g., L-proline) that are used in plant cell manipulation or for cellular protection against stress (Liang et al., 2013). Thus, the relatively short list of species which are known to be transformed by *A. tumefaciens* with the help of embryogenic cell suspension is extended by the three species of gentians studied here. It is noteworthy that although we produced many cell aggregates expressing *uidA* gene and showing resistance to kanamycin, and observed numerous structures indicative of advanced embryogenesis, our experiments did not produce a large number of T0 transformants; in fact, we regenerated only a few transformants. Thus, we would like to formulate a hypothesis that this relatively simple type of transformation involving a relatively noncomplex "classical plasmid" reduced the embryogenic potential of cell suspensions of the gentian species investigated. It was not a case of optimal post-transformation culture conditions, but rather a case of the numerous treatments that were associated with the transformation procedure, as well as the morphogenic potential of the cells. Somatic embryogenesis occurred more rarely in the cell suspensions investigated than in the wild-type suspensions. The results presented here support the recently published data concerning the nonvector protoplast transformation system. Based on the application of the same plasmid electroporation in the protoplast transformation of embryogenic cell suspensions of *G. kurroo* and regeneration of T0 transformants (Wójcik and Rybczyński, 2015), it appears that the embryogenic characteristics of gentians are very gentle and sensitive to their genome modifications.

## Conclusions

By using *A. tumefaciens* strain C58C1 carrying *nptII* and *uidA* genes, we transformed embryogenic cell lines of three species of gentians (*G. lutea*, *G. cruciata*, *G. kurroo*). Transgenic lines were selected using 50 $mg \cdot l^{-1}$ kanamycin and analyzed for β-glucuronidase expression. Only one of the three lines of *G. cruciata* showed the potential of somatic embryo regeneration. Some embryos were able to reach the germling stage and developed into T0 plantlets. All the experiments indicated that L-glutamine at a concentration 1.0 $g \cdot l^{-1}$ was crucial for improving the transformation efficiency. At the L-glutamine concentration of 2.0 $g \cdot l^{-1}$, a difference in the response of cell suspension of the studied species was observed. Taken together, we conclude that the *in vitro* embryogenesis of the studied gentians is very sensitive to genome modification and the treatments involved in the transformation procedure.

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**Authors' contributions**

AIW conducted the laboratory experiments. JJR wrote the manuscript.

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